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U.S. Environmental Protection Agency
Office of Pollution Prevention and Toxics
EPA East Building, Room 6428
1201 Constitution Avenue, NW
Washington, DC 20004
Attention: 8(e)

Contain NO CBI

Dear TSCA 8(e) Coordinator:

On behalf of the Alkylphenols & Ethoxylates Research Council, and its members,[1] I am writing to notify and provide the Agency with the draft Technical Report of an acute toxicity study of 4-Octylphenol in *Gammarus pulex* that we obtained from the United Kingdom's Environment Agency. The report entitled, "The acute toxicity of 4-Octylphenol to nymphs of the freshwater shrimp *Gammarus pulex,*" was apparently commissioned to investigate the acute toxicity of octylphenol to early instar *G. pulex* nymphs.

In this study, the test organisms were exposed to a range of 4-octylphenol concentrations for 96 hours under semi-static conditions. Survival and mobility were monitored over the exposure period. According to the report, "[B]ased on analysed concentrations of octylphenol, the 96-hour LC_{50} for *G. pulex* was 19.6 ug l^{-1} and the 96-hour EC_{50} (immobilisation) was 13.3 ug l^{-1}.

This study is being provided to the Agency as it appears that these findings may be reportable in accordance with EPA's TSCA Section 8(e) guidance.

Sincerely,

Robert J. Fensterheim
Executive Director

[1] Members of the Alkylphenols & Ethoxylates Research Council include: Dover Chemical Corporation; Rohm and Haas Company; Rhodia Inc.; Schenectady International, Inc.; and, The Dow Chemical Company.

MR287601

The acute toxicity of 4-Octylphenol to nymphs of the freshwater shrimp *Gammarus pulex*

WRc plc

Draft R&D Technical Report

The acute toxicity of 4-Octylphenol to nymphs of the freshwater shrimp *Gammarus pulex*

I Sims and P Whitehouse

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Draft R&D Technical Report

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EXECUTIVE SUMMARY

During the derivation of the proposed Environmental Quality Standards (EQS) for octylphenol, concerns were raised over the lack of toxicity data for this material to freshwater invertebrates. The effect of this was to introduce a high degree of uncertainty into any conclusions reached about an appropriate standard for octylphenol.

A comparison of effects data for octylphenol and the closely related nonylphenol indicates similar sensitivities of freshwater organisms to the two compounds and suggests that the same standard for both substances may be warranted. Previous experimental work conducted in support of the EQS for nonylphenol using early instar *Gammarus pulex* nymphs found that these were highly sensitive to this material, with a 96-hour LC_{50} of 24.6 ug l^{-1} and a 96-hour EC_{50} (immobilisation) of 12.7 ug l^{-1}. If *Gammarus* nymphs showed a similar sensitivity to octylphenol, then this would further strengthen the case for applying the same standard to octylphenol as that applied to nonylphenol.

A study was therefore commissioned by the Environment Agency to increase the data base on the aquatic toxicity of octylphenol to invertebrates. Specifically, the objective of the study was to investigate the acute toxicity of octylphenol to early instar *G. pulex* nymphs. The test organisms were exposed to a range of 4-octylphenol concentrations for 96 hours under semi-static conditions. Survival and mobility were monitored over the exposure period. Based on analysed concentrations of octylphenol, the 96-hour LC_{50} for *G. pulex* was 19.6 ug l^{-1} and the 96-hour EC_{50} (immobilisation) was 13.3 ug l^{-1}. As with the previous study using 4-nonylphenol, these data indicate high sensitivity to acute exposure to 4-octylphenol. In addition, they confirm very similar sensitivities to both compounds.

It would appear that the EQSs for 4-octylphenol may legitimately be set at similar levels as those for 4-nonylphenol.

It is recommended that the chronic toxic effects of this compound to *G. pulex* be investigated, for example by looking at its effects on feeding behaviour, as this is an ecologically important detritivorous invertebrate in many of the UK's river systems. The possibly that larvae and nymphs of other arthropods may be equally adversely affected must be considered. *Asellus aquaticus* occupies a similar ecological niche to *Gammarus* spp. in both lakes and rivers, but the effects of OP on this organism are unknown.

KEY WORDS

octylphenol, OP, nonylphenol, NP, *Gammarus pulex*, acute toxicity, EQS

1. INTRODUCTION

1.1 Octylphenol in surface waters

An important route of entry for octylphenol (OP) into water bodies is via effluents discharged from sewage treatment plants. OP may be formed during the aerobic biodegradation of octylphenol polyethoxylates which originate from the use of surfactants, industrial cleaning agents and other products. OP is resistant to subsequent anaerobic degradation and may eventually enter the recieving water in the final effluent. Sewage effluent discharged from a treatment works in Yorkshire was found to contain 0.5 ug l^{-1} OP but water in the outer Tees estuary has been reported to contain up to 13 ug l^{-1} total OP (Blackburn and Waldock 1995).

1.2 Toxicity to aquatic organisms

Limited aquatic toxicity data are available for OP. In acute toxicity tests, the only invertebrate for which data are available is *Daphnia magna*, for which the lowest reported effect concentration is a 24-hour EC_{50} of 170 ug l^{-1} (IUCLID 1996). data for several fish species are available and the lowest reliable value is a 96-hour LC_{50} of 250 ug l^{-1} for the fathead minnow (*Pimephales promelas*); OP is less toxic to algae (72h EC_{50} to *Scenedesmus subspicatus* of 1100 ug l^{-1}) and appears to be of low toxicity to bacteria (IUCLID 1996).

In vitro, OP has been found to be a more potent oestrogen mimic than the chemically similar nonylphenol (Jobling and Sumpter 1993, White *et al* 1994). Furthermore, concentrations of OP as low as 3 ug l^{-1} have been shown to induce vitellogenesis in rainbow trout (Jobling *et al* 1996), almost an order of magnituge lower than the lowest effective concentration for vitellogenesis with nonylphenol (NP).

1.3 Proposed Environmental Quality Standards for Octylphenol

Reflecting concerns about possible impacts of alkylphenols on aquatic life, the available aquatic toxicity data for both nonylphenol (NP) and OP have been reviewed. Whilst standards for NP have been proposed (Whitehouse *et al*, 1998), there are insufficient data to propose standards for OP for the protection of aquatic life. It has been suggested that this could lead to inadequate regulation of a potentially hazardous substance, especially if regulation of NP encouraged a shift toward use of OP. A comparison of toxicity data for species which have been tested with both NP and OP show a good correlation between the sensitivity of freshwater organisms to these compounds, suggesting that standards derived for NP may also be adequate for protecting aquatic life from the effects of OP.

A recent study (Sims *et al*, 1997) demonstrated high sensitivity of nymphs of the freshwater shrimp (*Gammarus pulex*) to NP, resulting in 96h EC_{50} and LC_{50} values of 12.7 ugl^{-1} and 24.6 ugl^{-1} , respectively. This was the critical data on which the current standards for NP have been based. It can be argued that, if *Gammarus* nymphs show a similar sensitivity to OP, then the case for applying the same standard to both alkylphenols is strengthened still further.

1.4 Aims of this study

The purpose of the study described here was to determine the acute toxicity of OP to *Gammarus pulex* and thereby determine whether or not there was supporting evidence for applying the same EQSs to both NP and OP.

The experimental conditions were designed to resemble those previously used in the study with NP (Sims *et al*, 1997). For this reason, animals from the same source and of similar size were employed, and a source of OP secured which was of a similar isomeric composition to the NP evaluated previously. Analytical confirmation of the exposure concentrations was also employed so that the LC_{50} and EC_{50} values could be calculated using the actual (analysed) concentrations.

2. TEST METHODS

2.1 Test substance

Technical grade 4-OP (99% pure) with a mixed (i.e. a combination of straight chain and branched chain) octyl moiety was obtained from Aldrich Chemicals (Lot No. 04210JN).

2.2 Test organisms

The test organisms used were from the same source as those used for the evaluation of NP. They were also held and fed in the same way and animals of a similar developmental stage were selected for testing.

First and second instar nymphs of the freshwater shrimp, *Gammarus pulex*. Adult *Gammarus* were collected from Watlington Brook (OS Map. Ref. SU 683 947) in October 1997 and maintained in culture under flow-through conditions until required for testing. No mortalities were evident during holding although it is likely that any dead animals would have been eaten by other *Gammarus*. The environmental conditions were as follows:

- Temperature - 13.2 to 14.4 °C
- pH - 8.0 to 8.2
- Dissolved oxygen (DO) - 86 to 92% of the air saturation value (ASV)
- Food, in the form of dead alder leaves, was supplied as required

On the day of the test, newly produced juvenile *Gammarus* were isolated from the stock tank by differential sieving. Animals passing a 600 um sieve but retained on a 250 um sieve were used in the experiment. These were first and second instar nymphs of approximately 2 mm in length.

2.3 Dilution water

The dilution water was groundwater with a total hardness of around 170 mg l^{-1} as $CaCO_3$ and a bicarbonate alkalinity of around 110 mg l^{-1} as $CaCO_3$.

2.4 Experimental design

2.4.1 Stability/solubility test

A preliminary test was carried out to determine the solubility and stability of OP under the test conditions.

Two aqueous concentrations of OP, 220 and 22 ug l^{-1}, were prepared, along with a groundwater control. A 1 mg ml^{-1} stock solution of the test material was prepared in acetone and used to prepare a 1000 ug l-1 working solution by adding 1 ml of the acetone stock solution to 0.5-l of distilled water in a 1-l volumetric flask. In order to achieve dissolution of the test substance in the working solution, the distilled water was heated to 80 °C and placed on an autostirrer operating at 1000 rpm before addition of the acetone stock solution. Dissolution was achieved and the whole was allowed to cool slowly while mixing. Once cool, the volume was adjusted to that required (1-l) using groundwater at the test temperature. Once analytical samples had been taken the solutions were placed in 250 ml beakers, covered with watchglasses and left for 24 hours in the test area. After this time a second set of samples were taken for analysis.

2.4.2 Acute toxicity study

A preliminary range-finding test was performed followed by a definitive semi-static test. The study was performed in an environmentally controlled facility with a photoperiod of 16 hours light, 8 hours dark and 30 minute simulations of dawn and dusk. The air temperature was maintained at around 14 °C. In both the range-finding and definitive tests, the test and control animals were exposed, unfed, for 96 hours.

The same procedure was used for preparing the exposure concentrations as that described above, except that the concentration of the working solution was 100 ug l^{-1} in both cases.

The following nominal concentrations of OP were used for the range-finding toxicity test:

0.0 (control), 0.01, 0.1. 1.0. 10 and 100 ug l^{-1}

The concentrations used for the definitive test were selected using the toxicity data from this preliminary range-finding test:

0.0 (control), 0.0 (acetone control), 5.6, 10, 18, 32 and 56 ug l^{-1}

The test vessels and volumes were the same as those used for the stability test. The test solutions were not aerated. Each treatment was replicated twice in the preliminary test and four times in the definitive test.

The animals were added to the test vessels until each vessel contained five animals, i.e. 10 animals were exposed at each treatment during the preliminary test and 20 for the definitive test. During both tests the test and control solutions were renewed every 24 hours by filling a duplicate set of vessels and transferring the animals to the fresh solutions using a large-bore pipette. Water quality measurements of temperature, pH and dissolved oxygen (DO) were made at the start and end of the tests on each treatment, and on old and new solutions at each renewal. Total hardness and bicarbonate alkalinity were measured using the new solutions from the control, 5.6, 18 and 56 ug l^{-1} treatments at the start of the definitive test and on the old solutions at the first renewal. For the preliminary test the same measurements were made on the control and the lowest (0.001 ug l^{-1}) and highest (100 ug l^{-1}) exposure concentrations.

The number of mobile, immobile and dead *Gammarus* were recorded for each vessel at two hours, and then at 24 hour intervals from commencement up to the end of the tests (96 hours).

2.5 Analysis of test solutions

2.5.1 Sampling of test solutions

For the stability test, a sample (~250 ml) was taken from each treatment solution and from the groundwater control immediately after preparation, and from the test vessels 24 hours later.

During the toxicity tests only new solutions were analysed as the results from the stability test showed that the test substance was stable over 24 hours (the inter-renewal period) under the environmental conditions used. Individual samples of new solutions (~250 ml) were taken for analysis of 4-OP on two occasions: at commencement of each toxicity test and on day four. For the preliminary test, only the 100 ug l^{-1} treatment (and control) was sampled (all other treatments were prepared from this) while for the definitive test all exposure treatments and the solvent control were sampled.

2.5.2 Sample Preparation Procedure

A 100 ml aliquot of each sample was spiked with 1 ug of internal standard (D10 phenanthrene) and acidified to pH 2 with 10% sulphuric acid. This was transferred to a separating funnel and shaken for 2 minutes with 100 ml of dichloromethane (DCM). The DCM was then separated into a flask and the extraction repeated with a further 20 ml of DCM which was also added to the flask. Water was removed by freezing and the sample concentrated to 1 ml under nitrogen. A blank and a spiked sample were analysed with each batch of test samples.

2.5.3 Analysis

The samples were analysed by gas chromatography (GC) and mass spectrometry using a PH 5890 GC directly coupled to a VG trio-1 mass spectrometer operating in the selected ion recording mode using +EI ionisation. The following ions were monitored:

m/z 77, 107, 188 and 206

The GC conditions were: 60 °C held for 4 minutes,

temperature increased to 300 °C, by 8 °C $min.^{-1}$ increments,

300 °C held for 11 minutes.

A series of calibration standards containing known concentrations of OP and the internal standard were run prior to the analysis.

3. RESULTS

3.1 Solubility/stability test

The analytical results for the solubility/stability test (Table 3.1) show that the initial concentrations were approximatley 70% of the nominal concentrations. After 24 hours under the environmental conditions to be used for the subsequent toxicity tests the concentrations had fallen by around 22% of the initial analysed concentrations. Clearly it was important to base estimates of toxicity on measured rather than nominal concentrations. Furthermore, a 24 hour replacement regime was required to maintain exposure concentrations.

Table 3.1 Analytical results for the solubility/stability test

Nominal concentrations (ug l^{-1})	Analysed concentrations at 0 hours (% of nominal)	Analysed concentrations at 24 hours (% of original)
0 (control)	< 5	< 5
22	15.1 (69)	11.9 (79)
220	153 (70)	116 (76)

3.2 Preliminary test

The ranges of water quality data recorded during the preliminary test were as follows:

temperature 13.8 - 14.9 °C

pH 7.62 - 8.21

DO 87 - 93 % ASV

Total hardness 122 - 218 mg l^{-1} as $CaCO_3$

Bicarbonate alkalinity 65 - 143 mg l^{-1} as $CaCO_3$

The large variations in total hardness and alkalinity were due to the method used for praparation of the test concentrations. The 100 ug l^{-1} treatment comprised a mix of 1:1 distilled water : groundwater (i.e. this was the 100 ug l^{-1} working solution) and so the resulting hardness of high test concentrations was greater than that of the lower concentrations.

The concentration of of 4-OP in the 100 ug l^{-1} nominal treatment, used to prepare the other treatments in this test, was 57 ug l^{-1} at the start of the test and 66.5 ug l^{-1} at 72 hours (fresh solutions). Hence all batches were probably around 60% of nominal.

At 96-hours all the animals exposed to 100 ug l^{-1} (nominal) 4-OP were incapable of swimming (immobile) and 70% of these were died (no sign of respiratory movements when viewed at x 7.5 magnification). 30% of the animals exposed to 10 ug l^{-1} (nominal) were immobile at this time and 20% had died. All animals in the remaining treatments were mobile at 96-hour exposure.

3.3 Definitive test

3.3.1 Water quality

The water quality data recorded during the study are summarised in Table 3.2. This shows that the parameters measured remained within acceptable limits throughout the study.

Table 3.2 Ranges of water quality recorded for the *Gammarus* acute toxicity test with OP

Parameter	Measured range
Temperature (°C)	13.4 - 15.8
pH	7.84 - 8.25
DO (%ASV)	90 - 97
Total hardness (mg l^{-1} as $CaCO_3$)	146 - 170
Bicarbonate alkalinity (mg l^{-1} as $CaCO_3$)	95 - 108

It is evident from these data that there was some variation in pH and temperature during the test. The maximum pH range, recorded from different vessels, was 0.41 pH units. Given the physico-chemical properties of OP, this degree of variation will have had little or no influence on toxicity. The variations in temperature and DO were within the range for OECD Guideline 203 ($\pm$ 2 °C, <30% DO). Total hardness and bicarbonate alkalinity were less influenced by the method used to prepare the test dilutions than was the case with the preliminary range-finding test. This was because the volumes of working solution used to prepare the treatments were less than those used in preparation of the test solutions used in the range-finding test.

3.3.2 Chemical analysis of test solutions

The exposure concentrations of 4-OP derived from the analytical data are shown in Table 3.3.

Table 3.3 Concentrations of 4-OP for the *Gammarus* acute toxicity test.

Nominal concentration of OP (ug l^{-1})	Measured concentrations of OP (ug l^{-1})		Mean concentration of OP (ug l^{-1})
	t_0	t_{72}	
0 (Control)	-	-	-
0 (Acetone control)	<5.0	<5.0	<5.0
5.6	8.9	8.8	8.9
10	11.0	10.8	10.9
18	15.4	15.5	15.5
32	31.3	23.0	27.2
56	46.1	55.2	50.7

These data show that measured test concentrations closely paralleled the nominal concentration series. Greatest discrepancy was seen at the highest concentration in the first set of samples analysed (t_0) where a difference of 20% was evident between the nominal and actual concentrations of OP. Generally close agreement between batches of test solutions was evident suggesting that exposure conditions over the course of the experiment were attained in a reproducible manner.

The concentrations determined in the two batches analysed were averaged to produce a geometric mean, and these means used for calculating the effect concentrations. The mean values will be used as the exposure concentrations throughout the remainder of this report.

3.3.3 Toxicity

The results for immobilisation and mortality are shown in Tables 3.4 and 3.5. At 96 hours all gammarids exposed to 50.7 and 27.2 ug l^{-1} were immobilised; all were dead at the higher concentration and 65% of those exposed to 27.2 ug l^{-1} were dead after this period. At this time, 75% were immobile in the 15.5 ug l^{-1} treatment (45% dead) and 20% were immobile at 10.9 ug l^{-1} (10% dead). Animals in the other treatments appeared to suffer no adverse effects.

At 48 hours, five animals were missing from two replicate vessels at 8.9 and 10.9 ug l^{-1} and from one replicate at 15.5 ug l^{-1}. They had been present at the renewal the previous day and it is presumed that they were eaten by the other animals in these vessels. One further animal from a different replicate at the 10.9 ug l^{-1} treatment had similarly disappeared by 72 hours. These losses would not have compromised the interpretation of the observed effects as they represented a very small proportion of the animals exposed. No losses were seen in the controls and all animals were accounted for at the higher concentrations.

Table 3.4 Immobile *Gammarus* exposed to 4-OP (percent affected).

Concentration (ug l^{-1})	Exposure period (hours)				
	2	24	48	72	96
0 (Control)	0	0	0	0	0
0 (Acetone control)	0	0	0	0	0
8.9	0	0	0*	0*	0*
10.9	0	0	0*	5#	20#
15.5	0	0	0+	35+	75+
27.2	0	0	45	70	100
50.7	0	5	80	95	100

+ = 1 missing, assumed eaten, at 48 hours. * = 2 missing, assumed eaten, at 48 hours, # = 1 missing, assumed eaten, at 72 hours

Table 3.5 Mortality of *Gammarus* exposed to 4-OP (percent affected).

Concentration (ug l^{-1})	Exposure period (hours)				
	2	24	48	72	96
0 (Control)	0	0	0	0	0
0 (Acetone control)	0	0	0	0	0
8.9	0	0	0*	0*	0*
10.9	0	0	0*	5#	10#
15.5	0	0	0+	25+	45+
27.2	0	0	20	45	65
50.7	0	5	25	50	100

+ = 1 missing, assumed eaten, at 48 hours, * = 2 missing, assumed eaten, at 48 hours, # = 1 missing, assumed eaten, at 72 hours

These data were used to estimate the concentrations required to kill 50% (LC_{50}) and immobilise 50% (EC_{50}) exposed gammarids at these times. The probit method (Finney 1971) was used utilising the generalised linear modelling functions and the probit link function in GENSTAT 5, release 3 (Payne 1993). In addition, the 95% confidence intervals around these

values were calculated (Finney 1971). The results of these calculations are shown in Tables 3.6 and 3.7. For the purposes of these calculations, the 6 animals that disappeared during the test were assumed to have survived.

Table 3.6 EC_{50}s (with 95% confidence intervals) for 4-OP to *Gammarus pulex*

24-hour	48-hour	72-hour	96-hour
-	30.5	21.2	13.3
-	(26.1 - 40.0)	(18.2 - 25.5)	(12.3 - 14.8)

Table 3.7 LC_{50}s (with 95% confidence intervals) for 4-OP to *Gammarus pulex*

24-hour	48-hour	72-hour	96-hour
-	74.8	40.0	19.6
-	(49.4 - 306)	(29.5 - 71.8)	(16.8 - 23.4)

4. DISCUSSION

Gammarus pulex plays an important role in aquatic ecosystems. It is a significant detritivore specialising in the shredding and breakdown of organic debris such as abscised leaves that fall into rivers and dead aquatic plant material. In so doing, it conditions this substrate for further breakdown by microbial attack, and the subsequent nutrient cycling that this leads to. As such, it may be regarded as a 'keystone' species in the food webs of many riverine systems.

4-OP exhibited a high acute toxicity to the nymphs of *G. pulex* tested in this study. This is a valuable piece of data, given the limited information that has been published on the ecotoxicology of this substance. The lowest published acute toxicity value for OP to an aquatic invertebrate is 170 ug l^{-1} (MATC) for *Daphnia magna* (IUCLID 1996). The EC_{50} for immobilisation of *Gammarus* found during this study is more than an order of magnitude lower than this (13.3 ug l^{-1}), illustrating the problems associated with limited data sets in EQS derivation. The data are also extremely close to the effects concentrations determined for NP to the same species under similar conditions (Table 4.1). Given the sensitivity of *Gammarus* nymphs to these substances and the very similar effects concentrations for both alkylphenols, the results obtained in this study and the previous study reported by Sims *et al* (1997) support the contention that standards for NP for the protection of aquatic life may be applied to OP also. The standards currently proposed for NP are a maximun allowable concentration (MAC) of 2.5 ugl^{-1} and an annual average (AA) of 1 ug l^{-1}.

Table 4.1 Comparison of NP and OP acute toxicity to nymphs of *Gammarus pulex*

	96h EC_{50} (immobilisation) (ugl^{-1})	96h LC_{50} (ugl^{-1})
4-Nonylphenol	12.7	24.6
4-Octylphenol	13.3	19.6

G. pulex is a riverine organism found in flowing water bodies. The organism occupying this ecological niche in still and slow-flowing waters is the isopod *Asellus aquaticus*. As such, *Asellus* is of high ecological significance for the same reasons as *Gammarus*. Unfortunately, nothing appears to be known of the effects of nonylphenol or OP on *Asellus*. In the light of the findings reported here, it would seem prudent to investigate the ecotoxicological effects of OP (and NP) to indigenous freshwater invertebrates further, in order to ensure that the proposed EQSs are adequate to protect this important class of organisms. Such investigations should include an assessment of their chronic toxicities to *Gammarus*, possibly using feeding rate as an end point, and also their acute toxicities to *Asellus aquaticus*. These data would remove some of the uncertainty surrounding the ecolotoxicological effects of OP and nonylphenol, and strengthen the basis for any EQSs set for these substances.

5. RECOMMENDATIONS

- Proposed EQSs for NP for the protection of aquatic life may also be applied to OP
- Further investigations of the ecotoxicological effects of nonylphenol and OP on freshwater amphipods are advised. These could include investigating the chronic effects of NP and OP on *Gammarus* feeding and the acute effects on *Asellus aquaticus*.

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